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Processing and secretion of the N-terminal domain of α-dystroglycan in cell culture media

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Abstract **α-Dystroglycan (α-DG) plays a crucial role in maintaining the stability of muscle cell membrane. Although it has been shown that the N-terminal domain of α-DG (α-DG-N) is cleaved by a proprotein convertase, its physiological significance remains unclear. We show here that native α-DG-N is secreted by a wide variety of cultured cells into the culture media. The secreted α-DG-N was both *N*- and *O*-glycosylated. Finally, a small amount of α-DG-N was detectable in the normal human serum. These observations indicate that the cleavage of α-DG-N is a widespread event and suggest that the secreted α-DG-N might be transported *via* systemic circulation in vivo.**

Keywords: Dystroglycan; Muscular dystrophy; Processing of protein; Glycosylation; Proprotein convertase

1. Introduction

The dystroglycan complex is composed of two proteins, α- and β-dystroglycan (α-and β-DG) which are encoded by a single gene [1]. α-DG is a highly glycosylated extracellular peripheral membrane protein and binds to several extracellular matrix proteins including laminin, agrin and perlecan [2–4]. In turn, the transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane [2,3]. α-DG is composed of three distinct domains; the N-terminal, mucin-like and C-terminal domains (Fig. 1). The N- and C-terminal domains exhibit globular structure and are connected by the central mucin-like domain which is highly glycosylated by *O*-linked sugar chains [5].

Functional defect of α-DG is implicated in the pathogenesis of several types of congenital muscular dystrophies, in which aberrant glycosylation of α-DG causes severe reduction of its laminin binding activity [6–11]. Aberrant glycosylation of α-DG is also demonstrated in a wide range of carcinoma cells, where it may lead to abnormal cell-extracellular matrix interactions and thus contribute to invasion and metastasis of cancer cells [12–15]. Moreover, some pathogens such as lymphocytic choriomeningitis virus, Lassa fever virus and *Mycobacterium leprae* utilize α-DG as a receptor to invade host cells [16,17].

Recently, it has been demonstrated that the N-terminal domain of α-DG (α-DG-N) interacts with Large, a putative glycosyltransferase mutated in the patients with congenital muscular dystrophy type 1D (MDC1D) [10], and then it is cleaved by a proprotein convertase (PC) called furin [18,19] (Fig. 1). However, the physiological significance of the proteolytic cleavage of α-DG-N remains elusive. In the present study, we generated a specific antibody against α-DG-N and characterized its processing in cultured cells.

2. Materials and methods

2.1. Antibodies

Rabbit polyclonal antibody was generated by immunizing New Zealand white rabbits with a peptide corresponding to the 30 amino acids, 141–170, in the N-terminal domain of human α-DG (NGSHIPQTSSVFSIEVYPEDHSDLQSVRTA). The antibody against α-DG-N was affinity purified using GST-α-DG-N (anti-α-DG-N, AP1528) (Fig. 1). Mouse monoclonal antibody against the sugar chain moieties of α-DG (anti-α-DG-sugar, IIH6), mouse monoclonal antibody against the C-terminal domain of β-DG (anti-β-DG), and rabbit polyclonal antibody against the C-terminal domain of human α-DG (anti-α-DG-C, AP1530) were described previously [2,20]. Affinity isolated rabbit anti-laminin was obtained from Sigma–Aldrich.

2.2. Cell culture

Mouse myoblast, C2C12, African green monkey kidney, COS-7, human breast adenocarcinoma, MCF7 and human prostate carcinoma, DU145 were purchased from American Type Culture Collection. Human cervix carcinoma, HeLa and human embryonic kidney, HEK293 were obtained from Human Science Research Resource Bank. Cells were plated on plastic non-coated or laminin-coated culture dishes (DB Bioscience) and cultured in Dulbecco's modified Eagle's medium, except that HeLa, MCF7 and DU145 were fed in Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. In the case of MCF7, 0.01 mg/ml of bovine insulin was added to the medium. For some cell lines, medium was replaced by a serum-free one 2 to 4 days before harvesting cells. In some experiments, cells were cultured in the presence of 200 μM of decanoyl-RVKR-CMK (Calbiochem), an inhibitor of PC, for 2 days. The culture medium was concentrated using Amicon Ultra-15 (Millipore) and the cells were lysed in sample buffer (65 mM Tris–HCl, pH 6.9, 3% SDS, 1% β-mercaptoethanol, 115 mM sucrose, 0.0004% Bromophenol blue). Materials were separated by 5–15% SDS–PAGE and then analyzed by Western blotting.

2.3. Lectin chromatography

The culture medium of C2C12 cells was incubated with lectin bound agarose, including concanavalin A (Con A), lentil lectin (LCA), wheat germ agglutinin (WGA), *Ricinus communis* agglutinin 120 RCA120), *Aleuria auranlia* lectin (AAL), peanut agglutinin (PNA), *Maackia*

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Abbreviations: DG, dystroglycan; α-DG-N, N-terminal domain of α-dystroglycan; PC, proprotein convertase

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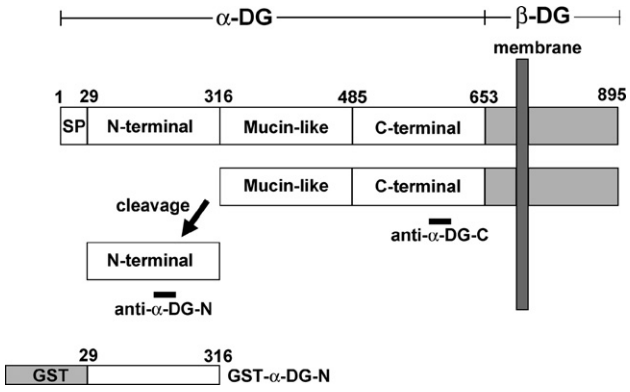

Fig. 1. Schematic representation of human α-DG and GST-α-DG-N fusion protein. Domain structures of α-and β-DG are shown. The N-terminal domain of α-DG is cleaved by a proprotein convertase. Positions of the epitopes for anti-α-DG antibodies are indicated by black bars. SP, signal peptide.

amurensis lectin (MAM), *Sambucus sieboldiana* agglutinin (SSA), phytohemagglutinin-E4, (PHA-E4) (Seikagaku Corporation) and Jacalin (Vector). The void fractions were analyzed by Western blotting using anti-α-DG-N.

2.4. Miscellaneous

For construction of a GST fusion protein, human *DAG1* gene (GenBank accession number NM 004393) corresponding to α-DG-N (amino acid 30–316) was amplified by PCR and subcloned into pGEX-2TK expression vector (GE healthcare) (Fig. 1). GST-α-DG-N fusion protein was expressed by *Escherichia coli* and purified as described previously [21]. Chemical deglycosylation was performed as described elsewhere [2,22]. Western blotting and laminin overlay assay were described previously [11]. Human sera were obtained from the members of the laboratory according to the standard protocol. Albumin concentration in the sera was reduced using ProteoSeek Albumin/IgG Removal Kit (Pierce).

3. Results

3.1. Characterization of the antibody against α-DG-N

We characterized the specificity of the antibody against α-DG-N (anti-α-DG-N) by Western blotting using GST-α-DG-N fusion protein. Anti-α-DG-N detected the 60 kDa band of GST-α-DG-N fusion protein (Fig. 2). Pre-absorption of the antibody by GST-α-DG-N fusion protein or antigen peptide eliminated the 60 kDa band completely (Fig. 2). On the other hand, pre-absorption of the antibody by GST protein or irrelevant control peptide did not eliminate the 60 kDa band (Fig. 2). The 60 kDa band was not detected by anti-α-DG-C, anti-α-DG-sugar, or anti-β-DG, while it was detected by anti-GST antibody. These data confirm the specific recognition of α-DG-N by this antibody.

3.2. Native α-DG-N is secreted into culture media by cultured cells

In the previous report, it was demonstrated that the overexpressed Fc-tagged α-DG was cleaved by a PC [18]. To see if native α-DG-N is cleaved by a PC and released from the cell surface, we analyzed the culture medium of C2C12 cells by Western blotting. Interestingly, anti-α-DG-N detected an intense and broad band with an apparent molecular mass of 37 kDa in the culture medium (Fig. 3). This band was eliminated completely by pre-absorption of the antibody by antigen

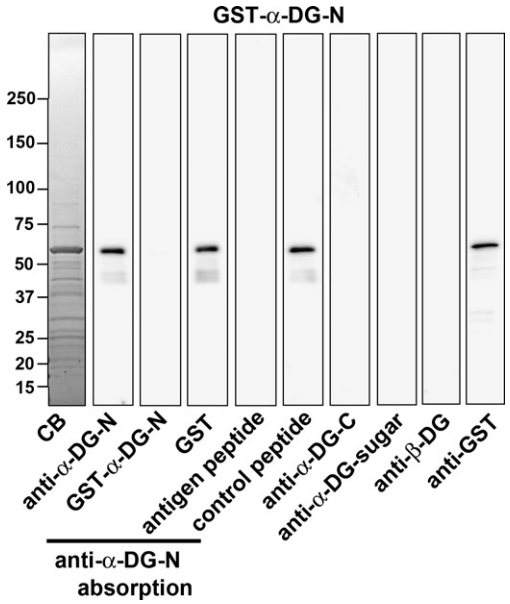

Fig. 2. Anti-α-DG-N recognizes the N-terminal domain of α-DG specifically. Anti-α-DG-N detected the 60 kDa band of GST-α-DG-N fusion protein. Pre-absorption of the antibody by GST-α-DG-N fusion protein or antigen peptide eliminated the 60 kDa band completely. Pre-absorption of the antibody by GST protein or irrelevant control peptide did not eliminate the 60 kDa band. The 60 kDa band was not detected by anti-α-DG-C, anti-α-DG-sugar or anti-β-DG, while it was detected by anti-GST antibody. Molecular mass standards ($D \times 10^3$) are shown on the left. CB, Coomassie blue staining of the gel.

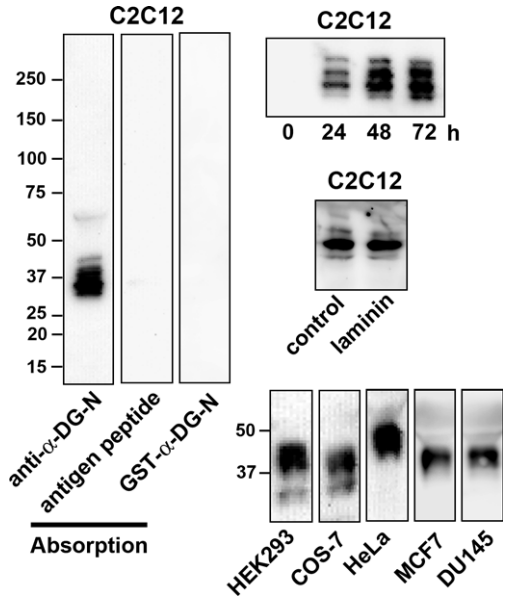

Fig. 3. Native α-DG-N is secreted into culture medium. The culture medium of C2C12 cells was analyzed by Western blotting using anti-α-DG-N. The antibody detected an intense and broad band of α-DG-N with an apparent molecular mass of 37 kDa, which was eliminated completely by pre-absorption of the antibody by antigen peptide or GST-α-DG-N fusion protein. The 37 kDa band increased progressively from 0 to 72 h of cell culture. There was no significant difference in the level of secretion of α-DG-N between the C2C12 cells cultured on the control plastic culture dishes and laminin-coated dishes. α-DG-N was also detected in the culture medium of a wide variety of cell lines, including HEK293, COS-7, HeLa, MCF7 and DU145. Molecular mass standards ($D \times 10^3$) are shown on the left.

peptide or GST-α-DG-N fusion protein (Fig. 3). Furthermore, the 37 kDa band increased progressively from 0 to 72 h of cell culture (Fig. 3). These results indicate that the 37 kDa protein is α-DG-N secreted from C2C12 cells into the culture medium. Closer inspection of the blots revealed that the broad 37 kDa band was composed of multiple bands with slightly different molecular masses (Fig. 3). To see if the interaction of α-DG with laminin may affect the secretion of α-DG-N, we also cultured C2C12 cells on the culture dishes coated with laminin. As shown in the right middle panel of Fig. 3, there was no significant difference in the level of secretion of α-DG-N between the control plastic dishes and laminin-coated dishes. We also examined if cell lines other than C2C12 secrete α-DG-N into culture medium. Western blotting demonstrated that a wide variety of cell lines including HEK293, COS-7, HeLa, MCF7 and DU145 secreted α-DG-N (Fig. 3). Interestingly, the molecular mass of α-DG-N varied considerably among different cell lines (Fig. 3). In particular, α-DG-N secreted by HeLa cells migrated around 45 kDa, slower than the other cell lines (Fig. 3).

3.3. Native α-DG-N is cleaved by a PC in C2C12 cells

To see if native α-DG-N is cleaved by a PC in C2C12 cells, we cultured C2C12 cells in the presence or absence of CMK, a PC inhibitor, and analyzed the cell lysate and culture medium by Western blotting. When the cells were cultured in the absence of CMK, α-DG with a molecular mass of 125 kDa was detected in the cell lysate by the antibody against the sugar chain moieties of α-DG (anti-α-DG-sugar, IIH6) and the antibody against the C-terminal domain of α-DG (anti-α-DG-C), as well as by laminin overlay assay (Fig. 4, upper panel). However, anti-α-DG-N did not react with this 125 kDa α-DG molecule (Fig. 4, upper panel). When the cells were cultured in the presence of CMK, anti-α-DG-N, anti-α-DG-sugar and anti-α-DG-C, as well as laminin overlay assay, all detected α-DG with a molecular mass of 160 kDa (Fig. 4, upper panel).

When the cells were cultured in the absence of CMK, a 37 kDa band was detected in the culture medium by anti-α-DG-N (Fig. 4, lower panel). However, this band was undetectable when the cells were cultured in the presence of CMK (Fig. 4, lower panel). The 37 kDa band was not detected in the cell lysate by anti-α-DG-N either in the absence or presence of CMK (Fig. 4, upper panel). These results indicate that native α-DG-N is excised by a PC and secreted into the culture medium immediately upon cleavage (Fig. 1). The secreted α-DG-N did not react with anti-α-DG-sugar and did not bind laminin in laminin overlay assay (Fig. 4, lower panel).

Fragments of α-DG suggestive of processing other than that of α-DG-N were not detected by anti-α-DG-C either in the cell lysate or culture medium (Fig. 4). As expected, the expression of β-DG in the cell lysate was not affected by CMK (Fig. 4, upper panel) and β-DG was not detected in the cell culture medium (Fig. 4, lower panel).

3.4. α-DG-N is both N- and O-glycosylated

To assess the status of glycosylation of α-DG-N, we performed enzymatic deglycosylation of cell culture medium. As described above, the molecular mass of α-DG-N was 37 kDa in C2C12 and 45 kDa in HeLa cell culture medium, respectively. Digestion by *N*-Glycosidase F decreased the molecular mass of α-DG-N by 5 kDa in both C2C12 and HeLa cell culture medium (Fig. 5A). On the other hand, digestion by neuraminidase decreased the molecular mass of α-DG-N by 3 kDa

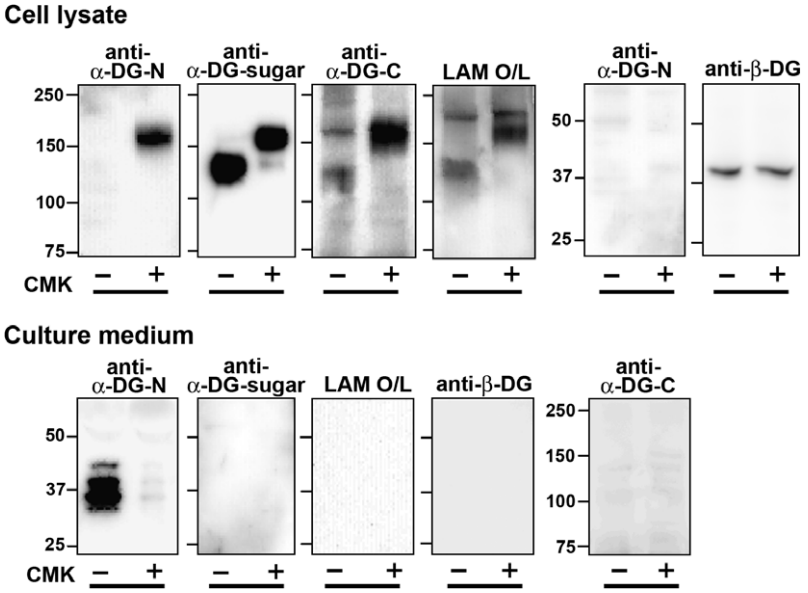

Fig. 4. Native α-DG-N is cleaved by a PC in C2C12 cells. C2C12 cells were cultured in the presence or absence of CMK, and then the cell lysate and culture medium were analyzed. When the cells were cultured in the absence of CMK, α-DG with a molecular mass of 125 kDa was detected in the cell lysate by the antibody against the sugar chain moieties of α-DG (anti-α-DG-sugar) and the antibody against the C-terminal domain of α-DG (anti-α-DG-C), as well as by laminin overlay assay (LAM O/L). Anti-α-DG-N did not react with the 125 kDa α-DG molecule. When the cells were cultured in the presence of CMK, anti-α-DG-N, anti-α-DG-sugar and anti-α-DG-C, as well as laminin overlay assay, all detected α-DG with a molecular mass of 160 kDa. When the cells were cultured in the absence of CMK, a 37 kDa band was detected in the culture medium by anti-α-DG-N. This band was not detectable when the cells were cultured in the presence of CMK. This 37 kDa band was not detected in the cell lysate when the cells were cultured either in the absence or presence of CMK. The 37 kDa α-DG-N in the culture medium did not react with anti-α-DG-sugar and did not bind laminin in laminin overlay assay. The expression of β-DG in the cell lysate was not affected by CMK and β-DG was not detected in the culture medium. LAM O/L, laminin overlay. Molecular mass standards ($D \times 10^3$) are shown on the left.

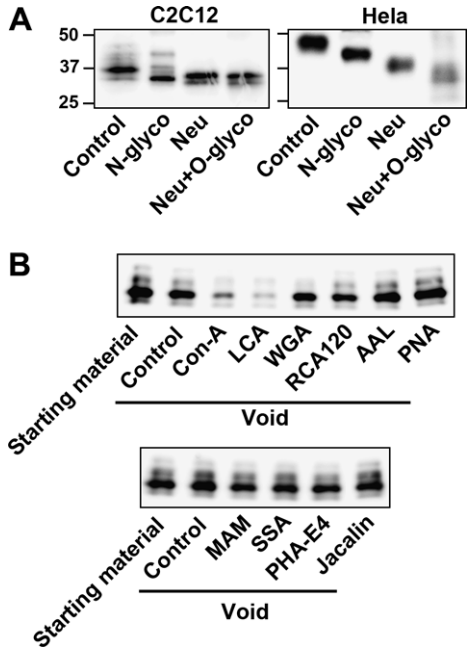

Fig. 5. α-DG-N is both *N*- and *O*-glycosylated. (A) The status of glycosylation of α-DG-N was analyzed by enzymatic deglycosylation. As described above, the molecular mass of α-DG-N was 37 kDa in C2C12 and 45 kDa in HeLa cell culture medium, respectively. Digestion by *N*-glycosidase F decreased the molecular mass of α-DG-N by 5 kDa in both C2C12 and HeLa cell culture medium. Digestion by neuraminidase decreased the molecular mass of α-DG-N by 3 kDa in C2 C12 and 8 kDa in HeLa cell culture medium, respectively. Digestion by neuraminidase together with *O*-glycosidase decreased the molecular mass of α-DG-N by 4 kDa in C2 C12 and 12 kDa in HeLa cell culture medium, to the final molecular mass of 33 kDa in both. Molecular mass standards ($D \times 10^3$) are shown on the left. (B) The sugar chain moieties of α-DG-N in the culture medium of C2C12 cells were analyzed by lectin column chromatography. The amount of α-DG-N remaining in the void fraction was decreased by incubation with Con-A and LCA column, but not other lectins.

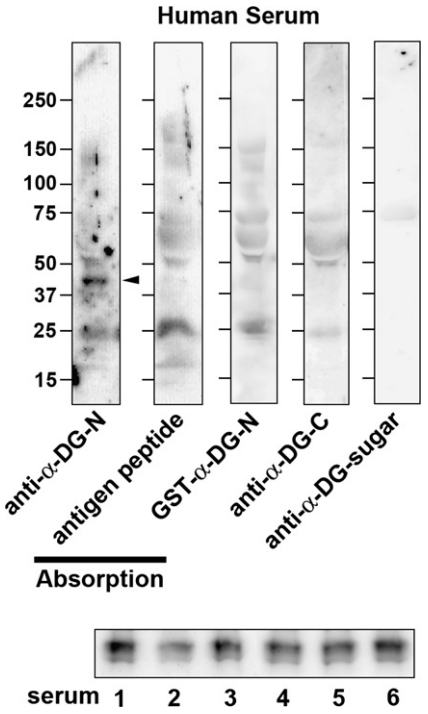

Fig. 6. α-DG-N is detectable in the normal human serum. Normal human sera were analyzed by Western blotting after depletion of albumin. Anti-α-DG-N detected a band with a molecular mass of 40 kDa (arrowhead), which was completely eliminated by pre-absorption of the antibody by antigen peptide or GST-α-DG-N fusion protein. This band was not detected by anti-α-DG-C or anti-α-DG-sugar. This 40 kDa band was detectable in all of the six other normal control sera. Molecular mass standards ($D \times 10^3$) are shown on the left.

in C2 C12 and 8 kDa in HeLa cell culture medium, respectively (Fig. 5A). Finally, digestion by neuraminidase together with *O*-glycosidase decreased the molecular mass of α-DG-N by 4 kDa in C2 C12 and 12 kDa in HeLa cell culture medium to the final molecular mass of 33 kDa in both (Fig. 5A). These data demonstrate that α-DG-N is both *N*- and *O*-glycosylated and indicate that differential glycosylation is at least partly responsible for the difference in its molecular mass between C2C12 and HeLa cell culture medium observed before deglycosylation. We further attempted to characterize the sugar chain moieties of α-DG-N in the culture medium of C2C12 cells by lectin column chromatography. The amount of α-DG-N remaining in the void fraction was decreased by incubation with Con-A and LCA column, but not other lectins (Fig. 5B). These results suggest that native α-DG-N may posses *N*-linked glycans of high-mannose type and *N*-linked glycans of hybrid type with or without $\alpha 1 \rightarrow 6$ linked fucose to the reducing terminal GlcNAc.

3.5. α-DG-N is detectable in the normal human serum

The aforementioned results raised an intriguing possibility that α-DG-N may be secreted in the human body fluid in vivo. To test this possibility, we analyzed normal human sera by Western blotting using anti-α-DG-N. When the whole sera were analyzed, detection of bands by Western blotting was severely hindered by the presence of huge amount of albumin (data not shown). So we reduced the albumin concentration of the sera before analysis as described in Section 2. Interestingly, anti-α-DG-N detected a band with a molecular mass of 40 kDa, which was completely eliminated by pre-absorption of the antibody by antigen peptide or GST-α-DG-N fusion protein (Fig. 6). In addition, this band was not detected by other antibodies, including anti-α-DG-C and anti-α-DG-sugar (Fig. 6). Altogether, these results indicated specific recognition of the band by anti-α-DG-N. Furthermore, the 40 kDa band corresponding to α-DG-N was detectable in all the normal human sera we tested (Fig. 6, lower panel). These results indicate that a small amount of α-DG-N is present in the normal human serum.

4. Discussion

In the previous report, TSA201 cells were transfected with Fc-α-DG fusion protein and then cultured in the presence or absence of CMK [18]. The overexpressed fusion protein was purified by protein A beads and analyzed by Western blotting [18]. Fc-α-DG fusion protein did not react with the antibody against the N-terminal domain of α-DG when the cells were cultured in the absence of CMK [18]. When the cells were cultured in the presence of CMK, however, Fc-α-DG fusion protein migrated slower and became reactive with the antibody

[18]. In another report, the EpH4 cells were cultured in the presence or absence of CMK and the surface membrane proteins were biotinylated, immunoprecipitated by anti-β-DG antibody and then analyzed by Western blotting using streptavidin-HRP [19]. When the cells were cultured in the presence of CMK, α-DG which was co-immunoprecipitated with β-DG migrated slower and a 37 kDa band appeared upon digestion of this co-immunoprecipitates by furin, suggesting that the 37 kDa band might represent the N-terminal domain of α-DG [19]. Although these observations indicate that α-DG-N is cleaved by a PC, the native molecule of α-DG-N has not yet been identified using specific antibodies and it remains elusive whether the excised α-DG-N is secreted from cells or retained at the cell surface *via* interaction with certain cell surface molecules. In addition, it remains unclear if this proteolytic cleavage occurs in a wide variety of tissues ubiquitously or in limited tissues under limited conditions in vivo. In the present study, using a specific antibody against α-DG-N, we have demonstrated for the first time that native α-DG-N is secreted from the cultured cells into the culture medium immediately after cleavage by a PC. In addition, secretion of α-DG-N was observed in a wide variety of cell lines, suggesting that it might be a widespread event in vivo. This is consistent with the findings that both α-DG and furin are expressed ubiquitously [1,23].

A recent study on the crystal structure of recombinant α-DG revealed autonomous domains in α-DG-N [24]. The structure is characterized by an Ig-like domain and a domain resembling ribosomal RNA-binding proteins, connected by a long and flexible linker [24]. α-DG-N contains a consensus sequence for *N*-glycosylation at amino acid 139 [25]. By enzymatic deglycosylation, we have shown that α-DG-N in the culture medium of C2C12 or HeLa cells is actually *N*-glycosylated. Moreover, lectin chromatography suggested that α-DG-N is modified by *N*-linked glycans of high-mannose type and hybrid type. The mucin-like domain of α-DG is well known to be extensively and differentially *O*-glycosylated. Interestingly, our results of enzymatic deglycosylation indicate that α-DG-N is also modified by *O*-linked glycans differentially. Among the *O*-linked glycans of α-DG, *O*-mannosyl glycans play a critical role in the binding with laminin [26]. As judged from the results of laminin overlay assay, however, α-DG-N does not seem to possess the *O*-mannosyl glycan structure involved in the binding of laminin.

In the present study, we have shown that a 40 kDa band corresponding to α-DG-N is detectable in the normal human serum after depletion of albumin. The results indicate that a small amount of α-DG-N is present in the normal human serum and raise a possibility that α-DG-N might be secreted in the human serum in vivo. One of the important roles of α-DG-N is interaction with Large [18]. Mutant α-DG molecule lacking the N-terminal domain from the beginning does not bind laminin, because it can not interact with Large and thus glycosylation necessary for laminin binding does not proceed in the mucin-like domain [18]. However, the cleavage of α-DG-N itself does not seem to be essential for the function of α-DG in terms of laminin binding activity, because inhibition of cleavage by CMK dose not affect the laminin binding activity of α-DG itself [18]. In contrast, other substrates for furin, including growth factors, hormones or receptors, are initially synthesized as inactive proproteins, which are then processed to become the biologically active mature proteins by proteolytic cleavage [23]. Taking these observations into account, it is possible that the secreted α-DG-N, but not the membrane bound α-DG consisting of mucin-like and C-terminal domains, may be activated and new functional roles may be conferred by this cleavage. It should be noted, in this respect, that recombinant α-DG-N was shown to promote neurite extension of PC 12 cells [27]. In the present study, we have demonstrated the presence of a small amount of α-DG-N in the normal human serum. One intriguing possibility is that the secreted α-DG-N might be transported via systemic circulation and might exert hitherto unidentified effects at unidentified target organs in vivo. Another intriguing possibility is that the amount of α-DG-N in the serum might be altered in certain neuromuscular disorders such as α-dystroglycanopathy. Further studies are needed to clarify these points.

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